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**Liposome Mediated-CYP1A1 Gene Silencing Nanomedicine Prepared
Using Lipid Film-Coated Proliposomes as a Potential Treatment Strategy
of Lung Cancer**

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64

65 **ABSTRACT:**

66 The occurrence of lung cancer is linked with tobacco smoking, mainly through the
67 generation of polycyclic aromatic hydrocarbons (PAHs). Elevated activity of cytochrome
68 P4501A1 (CYP1A1) plays an important role in the metabolic processing of PAHs and its
69 carcinogenicity. The present work aimed to investigate the role of CYP1A1 gene in PAH-
70 mediated growth and tumor development *in vitro* and using an *in vivo* animal model. RNAi
71 strategy was utilized to inhibit the overexpression of CYP1A1 gene using cationic
72 liposomes generated using a lipid film-coated proliposome microparticles. Treatment of
73 PAH-induced human alveolar adenocarcinoma cell line with cationic liposomes carrying
74 CYP1A1 siRNA resulted in down regulation of CYP1A1 mRNA, protein as well as its
75 enzymatic activity, triggering apoptosis and inhibiting multicellular tumor spheroids
76 formation *in vitro*. Furthermore, silencing of CYP1A1 gene in BALB/c nude xenografts
77 inhibited tumor growth via down regulation of CYP1A1 expression. Altogether, our
78 findings showed that liposome-based gene delivery technology is a viable and stable
79 approach for targeting cancer causing genes such as CYP1A1. This technology facilitated
80 by the use of sugar particles coated with lipid films has demonstrated ability to generate
81 anticancer effects that might be used in the future for therapeutic intervention and treatment
82 of lung cancer.

KEYWORDS: Apoptosis, Cancer, CYP1A1, Lung, siRNA, Smoking, Tobacco

1. INTRODUCTION

Lung cancer has become a leading cause of death worldwide due to the increased environmental contamination with inhalable carcinogens occurring as byproducts of combustion processes and unhealthy habits such as tobacco smoking (Field and Withers, 2012). Despite the efforts made to improve the life quality of cancer patients, a proper understanding of the pathogenesis of lung cancer is still missing, resulting in poor treatment outcomes and severe adverse effects of chemotherapy and radiotherapy (Brambilla and Gazdar, 2009). Susceptibility of lung to carcinogenesis is based on the metabolic imbalance between induction and detoxification pathways, with a significant role of external inducing factors (Hecht, 1999).

Polycyclic aromatic hydrocarbons (PAHs) produced by tobacco smoking are involved in the activation and development of lung cancer (Armstrong et al., 2004; Hecht, 1999). Although the detailed mechanism of how this group of carcinogens disrupts the homeostasis of lung cells is still unclear, studies have concluded that PAHs can induce the overexpression of cytochrome P4501A1 gene (CYP1A1), an important member of a large family of cytochrome P450 enzymes involved in the metabolism of PAHs (Shimada and Fujii-Kuriyama, 2004). Consequently, many highly electrophilic metabolic intermediates

can be produced, causing irreversible damage to human tissues and inducing cancer occurrence (Shimada and Fujii-Kuriyama, 2004). Therefore, targeting of CYP1A1 gene may be a promising therapeutic strategy especially for smoking-related lung cancer (Androutsopoulos et al., 2009; Bruno and Njar, 2007). The induction of CYP1A1 primarily occurs when the inducer binds to the ligand-activated transcriptional factor aryl hydrocarbon receptor (AhR) (Guigal et al., 2000). Flavonoid galangin, an antagonist against AhR, has been considered as an inhibitor candidate to decrease the CYP1A1 expression (Ciolino and Yeh, 1999). However, multi-targeted properties of this drug may lead to non-specific inhibition of the other members of the P450 gene family (Murakami et al., 2008; Sak and Everaus, 2015), suggesting a better and specific strategy is needed to target CYP1A1 gene for therapeutic intervention and treatment of lung cancer.

RNA interference (RNAi) is a gene silencing technology at the transcriptional level and works through specifically targeting mRNA via sequence-specific matches, resulting in degradation of the target mRNA (Agrawal et al., 2003). siRNA technology promises greater advantages over conventional drugs currently in the market for its high targeting selectivity and low toxicity; however, pharmacokinetic properties of siRNA are unpredictable and its cellular uptake is poor (Lorenzer et al., 2015). Accordingly, specific

siRNA-mediated silencing of CYP1A1 expression with improved kinetics and uptake by target cells is urgently warranted.

As widely used vehicles in nucleic acid delivery, non-viral vectors such as cationic liposomes are much safer than viral vectors (Khurana et al., 2013). Furthermore, compared to polymeric vectors, cationic liposomes may offer higher transfection and greater biocompatibility (Ruozi et al., 2003). Novel cationic lipids conjugated with functional targeting groups may offer a great potential for use in the preparation of cationic liposomes (Ruozi et al., 2003; Kim et al., 2010b; Sun et al., 2018).

Liposomes manufactured using the traditional thin-film hydration technique with subsequent preparation as liquid dispersions are unstable during storage owing to the liability of phospholipids to hydrolysis and oxidation, with subsequent compromise of the validity of liposomes as drug carriers (Grit and Crommelin, 1993). Alternatively, proliposomes are stable powdered phospholipid formulations prepared by coating carbohydrate carrier particles with thin phospholipid films using modified rotary evaporators (Elhissi et al., 2006; Gala et al., 2015). Liposomes can be generated from proliposomes via the addition of aqueous phase and shaking (Elhissi et al., 2006; Gala et al., 2015). Several reports have established the suitability of manufacturing thin-film-based proliposome powders on a large scale, for instance by using fluidized-bed coating (Chen and Alli, 1987; Kumar et al., 2001; Gala et al., 2015). Liposomes generated from lipid film

coated sugars (i.e. proliposomes) have been widely investigated for drug delivery. For example, early reports have shown that oral delivery of non-steroidal anti-inflammatory drugs in liposomes generated from proliposomes can protect against gastric ulceration in experimental animals (Katare et al., 1990). Proliposomes have also been investigated for nasal delivery of propranolol hydrochloride and nicotine (Ahn et al., 1995; Jung et al., 2000a), and for parenteral administration of antifungal drugs (e.g. amphotericin B) (Payne et al., 1987), and anticancer agents such as methotrexate (Park et al., 1994) and doxorubicin (Wang et al., 2000), and for transdermal delivery of nicotine (Hwang et al., 1997; Jung et al., 2000b). We have previously shown that proliposomes made by coating sucrose with lipid films can generate inhalable liposomes when hydrated *in situ* within medical nebulizers (Elhissi et al., 2012). More recent investigators have shown that diltiazem HCL liposomes generated from proliposomes could be used for topical treatment of glioma (Mokhtar Ibrahim et al., 2013) and dermatitis (Jahn et al., 2014) using animal models. Proliposomes made by film-coating of sugar particles have recently been demonstrated to be compressible into tablets, with properties being dependent on formulation (Khan et al., 2018).

In this study, lipid film-based proliposome technology was employed for the preparation of cationic liposomes-siRNA (CL-siRNA) formulations for targeting the CYP1A1 gene. AhR-

mediated induced expression of CYP1A1 in A549 adenocarcinoma cell line was used to model smoking induction of CYP1A1 expression. The effects of CYP1A1 silencing with CYP1A1 CL-siRNA on CYP1A1 expression, CYP1A1 enzyme activity, cell apoptosis and tumor spheroids formation were verified in induced A549 cell lines. The effect of CYP1A1 silencing on tumor regression was further investigated in the induced A549 tumors in xenograft BALB/c-nude mice.

2. MATERIALS AND METHODS

2.1. Materials

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dioleoyl-phosphatidylethanolamine (DOPE) were purchased from Avanti Polar-Lipids Inc. (Alabaster, AL, USA). Cholesterol was obtained from Biotech Co. Ltd (Shanghai, China), and 3-methylcholanthrene (3-MC) was purchased from SUPELCO Co. (Pennsylvania, USA). Human CYP1A1 siRNA was chemically synthesized and purified via HPLC by RiboBio (Guangzhou, China). Goldview staining was purchased from Guangzhou Geneshun Biotech Ltd (Shanghai, China). RNA prep pure cell kit was purchased from TIANGEN (Beijing, China). The sequence for siRNA was as follows: siRNA against CYP1A1: sense, 5'-GGCCUGAAGAAUCCACCAG-3'; antisense, 3'-

CUGGUGGAUUCUUCAGGCC-5'. FAM-siRNA and the same sequence was obtained from Sangon Biotech (Shanghai, China). Lipofectamine2000 was obtained from Invitrogen (USA).

2.2. Preparation of liposome-siRNA complexes using lipid-coated particulate-based proliposomes

Sorbitol particles (300-500 μm) were placed in 50 ml pear-shaped flask and attached to a modified rotary evaporator with a feed-line tube. The flask was partially immersed in a water bath (37°C). A chloroform solution containing DOTAP, DOPE and Cholesterol (3:4:3 mole ratio) was injected in portions (0.5 mL each) via the feed-line using a syringe and by releasing the vacuum for a few seconds using a valve fitted on top of the condenser to allow lipid solution to be drawn through the feed-line and be sprayed onto the sorbitol carrier particles. After each addition, complete evaporation of chloroform was allowed before injecting the next portion. After solvent was completely evaporated, the solid particles of proliposomes were collected and stored in glass vials in the freezer (-18°C). Proliposomes were hydrated with water to form liposomes (1 mg/mL) followed by probe-sonication. The sonicated cationic liposomes (CL) were mixed with siRNA in RNase-free water using vortex-mixing and incubated for 30 min at room temperature to form CL-siRNA complexes.

2.3. Scanning electron microscopy (SEM) of lipid film coated proliposomes

Microparticles made by coating sorbitol carrier with lipid film were positioned onto a carbon pad (Agar Scientific, UK), and coated with a thin film of gold using the sputter coater of the microscope (Bio-Rad, England). The morphology of the resultant microparticles was investigated under vacuum using the Quanta 200 scanning electronic microscope.

2.4. Size analysis and zeta potential studies of CL-siRNA

Size analysis and zeta-potential studies of CL and CL-siRNA complexes were conducted using Photon Correlation Spectroscopy (PCS) and laser Doppler velocimetry, respectively. The studies were performed using the Malvern ZetaSizer Nano ZS90 (Malvern Instruments Ltd, UK) upon selecting the right software for each type of analysis. Size and size distribution were expressed by the instrument as the mean hydrodynamic diameter and polydispersity index (PDI), respectively.

2.5. Cell Culture Studies

Human alveolar adenocarcinoma, A549 lung cancer cell line was obtained from American Type Culture Collection (Rockville, MD, USA). A549 cell line was cultured in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Minhai, China), 100

U/ml penicillin, 100 mg/ml streptomycin. Cell culture was performed in an incubator maintained at 37°C in a humidified environment containing 5% CO₂.

2.6. Agarose gel retardation assay

To confirm formation of the complexes, the agarose gel retardation assay was employed to select the optimal charge ratio between cationic liposomes and negatively charged siRNA. CL-siRNA complexes were prepared at various molar ratios, and then run through a 2% agarose gel. The mobility of siRNA complexed with cationic liposomes was visualized by GoldView staining.

2.7. RNase protection assay of siRNA in cationic liposome complexes

An ideal siRNA delivery system is expected to protect siRNA against RNase enzymatic degradation. In order to monitor siRNA degradation by nuclease, firstly, siRNA-CL complexes were prepared at a final siRNA concentration of 5 µM and then incubated in the presence of 0.1mg/ml RNase for 30 min at 37°C. An aliquot (20 µl) was removed and snap-frozen at -80°C at every time point. All samples were thawed on ice when they were collected and immediately mixed with 5 µl of a 100 mM Triton X100 solution and 25 µl of RNA-extraction mixture (phenol/chloroform/isoamyl alcohol; 25:24:1). The siRNA was

precipitated with ethanol, electrophoresis was performed on agarose gel (2%) and visualization took place by GoldView staining (Buyens et al., 2008).

2.8. Cell model with high expression of CYP1A1

For the purpose of simulating the gene induction pathway to obtain a cell model with high CYP1A1 gene expression, an induction assay was carried out on A549 cells which are common for CYP1A1 gene research (Fazili et al., 2010). Cells were seeded in 12-well plates at a density of 1×10^4 cells per well, followed by 24 h incubation at 37°C in a humidified environment containing 5% CO₂, and cells were treated with 3-MC with a final concentration of 5 µM for further 24 h (3-MC was dissolved in DMSO). 3-MC is one of the most potent PAH carcinogens, which is usually used in the induction of CYP1A1 via the AhR mechanism (Abdelrahim et al., 2003). After the induction, the induced cells were collected and used in the subsequent experiments.

2.9. Cellular uptake of siRNA in induced A549 lung cancer Cells

Transfection of FAM-siRNA (what is FAM-siRNA) was performed in induced A549 cells. The induced A549 cells were seeded at a concentration of 5×10^5 cells per well in six-well plates. The cells were grown to a confluency between 60% and 80% and washed with pre-

warmed (37°C) PBS, and then they were incubated with 100 nM liposome-free FAM-siRNA or 100 nM FAM-siRNA-liposome complexes in serum-free medium. Following incubation for 4 h, the medium was replaced and the cells were washed with PBS twice, and then analyzed using flow cytometry (Beckman Coulter, USA) and examined under a fluorescence microscope. siRNA complexed with Lipofectamine2000 (Lipo2000) was used as a positive control in the experiments.

2.10. Silencing of 3-MC induced CYP1A1 gene in A549 lung cancer cell line

For evaluation of the mRNA of CYP1A1 gene *in vitro* and *in vivo*, RNA was extracted from cells 24 h after transfection with CYP1A1-specific siRNA (n = 3) or from A549 lung tumor (n = 3), respectively, using RNA prep Pure cell kit. cDNA was then obtained by reverse transcription of the total RNA using the TIANscript RT kit and the CYP1A1 (sense, 5'-GGCCUGAAGAAUCCACCAG-3'; antisense, 3'-CUGGUGGAUUCUUCAGGCC-5'). mRNA levels were analyzed using the SosoFast™ EvaGreen Supermix on iCycler iQ™ 5 system (Bio-Rad, USA) and β -actin was used as internal control. The PCR reaction was conducted at 95°C for 3 min followed by 40 cycles of 95°C for 5 s, and 56°C for 10 s in the iQ™5 Real-Time PCR Detection System. The expression of CYP1A1 was analyzed and normalized using the $2^{\Delta Ct}$ method relative to the expression of β -actin.

2.11. CYP1A1 enzyme assays

To further study the silencing effect of siRNA on CYP1A1, the enzyme activity as well as the content of CYP1A1 was assessed. The CYP1A1 enzyme activity was determined by Human CYP1A1 fluorescence quantitative detection kits (Genmed Scientifics INC.USA). The CYP1A1 enzyme content was measured using Human CYP1A1 ELISA kits (R&D systems, USA). Both assays were performed following the relevant suppliers' instructions.

2.12. Apoptotic assays

To examine the interactions between CYP1A1 gene regulation and the induced growth of tumor cells, different groups were designed in the cell apoptosis experiment. Induced A549 cells were treated with CYP1A1-specific siRNA (100 nM) or complexed with cationic liposome in serum-free medium for 4 h and then further incubated in fresh completed medium. Cells were washed with PBS and digested in trypsin for suspension after incubation for 48 h, followed by double staining with FITC-Annexin V and propidium iodide using the cell apoptotic analysis kit (Beyotime, China) following the manufacturer's instructions. Flow cytometry was used for investigation of cell apoptosis ($n = 3$). Further studies were performed to investigate the apoptosis mechanism. Caspases are the critical proteins responsible for apoptosis. These proteins are classified as initiators or executioners depending on their point of entry into the apoptotic cascade. It has been confirmed that

there were two main apoptosis pathways mediated by caspases (Boatright and Salvesen, 2003). Among all the family members in this pathway, caspase 3 was considered as the final executioner, and meanwhile, caspase 8 and caspase 9 are the key initiator proteins which exist in the extrinsic and intrinsic apoptotic pathways, respectively. The three caspases were firstly detected with Caspase Activity Assay Kits (Beyotime, China).

2.13. Multicellular tumor spheroids (MCTSs) assays

Multicellular tumor spheroids (MCTSs) may provide an appropriate model to identify the drug effect *in vitro* for its similarity to the tumor formation *in vivo* (Friedrich et al., 2009). A549 cells were cultured in a modified tumor sphere medium. The medium is comprised of recombinant fibroblast growth factor (EGF) (10ng/ml), basic fibroblast growth factor (bFGF) (10ng/ml) and insulin (4U/L), and plated at a density of 2×10^3 cells per well in 6-well plates. Spheres were formed after 8-10 days incubation. After 24 h 3-MC induction, spheres were treated with different groups of siRNA which were described in the gene silence study at a siRNA final dose of 100 nM for 4 h. After further 72 h incubation, the results were observed by microscope.

2.14. In vivo efficacy of targeting CYP1A1 gene using liposomes generated from lipid film-coated proliposomes

The animal study protocol was approved by Institutional Animal Care and Use Committee of the Sichuan University in China. Male BALB/c nude mice (weighing 20-23 g) were used to investigate the antitumor efficacy of targeting CYP1A1 gene *in vivo*. Briefly, 1×10^7 A549 cells were re-suspended in 200 μ l serum-free RPMI 1640 medium and injected subcutaneously into the right flank of the nude mice. After 5 weeks tumor-bearing mice were randomly divided into four treatment groups (5 animals each). At days 1, 4, 7, 10, 13 and 16, mice were intratumorally injected with 100 μ l 10% 3-MC solution. Then at days 2, 5, 8, 11, 14 and 17, mice were intratumorally injected again but with PBS, free siRNA or CL-siRNA. Every treatment was based on the dose of 40 μ g siRNA per mouse. Calipers were used in this work to measure the tumor progression of every mouse. Tumor volumes were calculated as $\text{length} \times \text{width} \times \text{width} \times 0.5 (\text{mm}^3)$. At the day 18, three animals from each group including control were sacrificed, and the tumors were excised. The measurements of CYP1A1 gene silencing effect were conducted as described earlier.

2.15. Statistical analysis

Values were presented as mean (\pm SD) unless otherwise stated. The differences between groups were analyzed using the Student's *t*-tests and one-way analysis of variance (ANOVA) with Bonferroni tests for multiple-group analysis. A probability level of $P < 0.05$ was considered to indicate significant difference between the groups.

3. RESULTS

3.1. Physical characterization of proliposomes, cationic liposomes (CL) and CL-siRNA complexes

The surface morphology of proliposome powders prepared through coating sorbitol particles with lipid film was examined by scanning electron microscopy (SEM) (Figure 1). The high porosity of sorbitol (Figure 1a) facilitated coating of the lipid on the carrier surfaces (Figure 1b). Our SEM observations using cationic lipids to coat sorbitol particles is in concordance with the previous findings using neutral lipids such as dimyristoylphosphatidylcholine coated onto sorbitol particles (Payne et al., 1986). Our study also further confirms that sorbitol is a highly suitable carrier for coating with lipid films and preparation of proliposomes because of its microporous structure. In another study, we demonstrated that the film coating proliposome technology can be scaled up using fluid-bed coating equipment that can deposit a lipid film on carbohydrate particles (e.g. sucrose), generating liposomes that can successfully entrap conventional small molecules, such as the antiasthma steroid beclometasone dipropionate (Gala et al., 2015). In

the present investigation, through a smaller scale of manufacturing using a modified rotary evaporator equipped with a feed tubeline, proliposomes made by coating sorbitol with cationic lipids were prepared. Upon hydration (including or excluding siRNA) and probe-sonication, cationic liposomes were generated. The measured size of the siRNA-free vesicles was as small as 85 ± 3.2 nm and the size distribution, expressed by PDI, was as low as 0.165. The uniform coating of sorbitol particles (Figure 1b) justifies the facilitated generation of liposomes in the nano-size range and the narrow size distribution (i.e. low PDI) (Figure 1d). Transmission electron microscopy (TEM) images confirmed the uniform round shape of the gene-free cationic liposomes, which were also similar to those incorporating siRNA, suggesting that the genetic material was complexed with the liposomes, with no apparent formation of siRNA aggregates (Figure 1c). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) in the formulation conferred the liposomes with a positive surface charge of about +43 mV (sorbitol solution pH=7.5) (Figure 1d). For formulations incorporating siRNA, the integrity of siRNA was studied (Figure 1e). Varying charge ratios of CL to siRNA (N/P ratio) were prepared at fixed siRNA concentration (100 nM). With the N/P ratio higher than 4, the migration of siRNA was completely retarded, indicating good binding efficiency of CL with siRNA and successful formation of the complexes (Figure 1e, f). On the other hand, size and zeta potential of CL-siRNA complexes were 90 nm and +30 mV, respectively at the N/P mole ratio of 4:1 (Figure 1d),

which contributed to the good dispersion properties and stability of CL-siRNA complexes.

Considering all the results above, N/P = 4:1 was chosen as the optimal charge ratio for CL-siRNA complex formation.

Agarose gel assay is an established technique for checking the formation of complexes between liposomes and genetic materials (e.g. siRNA) (Kim et al., 2010a). To assess the ability of liposomes to protect siRNA from degradation, the stability of siRNA in RNase solution was tested. As shown in Figure 1f, free siRNA was completely degraded upon exposure to RNase. By contrast, when siRNA was incorporated into cationic liposomes, the genetic material was intact for at least 4 h, indicating that liposomes have provided short-term protection for siRNA against enzymatic degradation. In this study, we made powdered cationic formulations of proliposomes by film coating the sorbitol sugar with cationic lipid. This can readily generate liposomes complexing with siRNA via addition of aqueous phase and sonication just on the day of administration; hence, storage instability of liposome dispersions is avoided.

3.2. Stimulation of CYP1A1 gene expression by 3-MC treatment in A549 lung cancer cell line

After incubation with 3-MC (5 μ M) for 24 h, the induced A549 cells were collected to investigate the target gene CYP1A1 expression level. All samples were analyzed by RT-PCR, which suggested that the mRNA level of CYP1A1 in induced cells was about 7 times higher than that in the normal cells. Enhanced CYP1A1 expression was maintained for at least 48 h after single induction.

CL were compared with Lipo2000, a commonly used positive control for siRNA delivery, for evaluation of the siRNA delivering ability. FAM-labeled siRNA was prepared alone or mixed with CL or Lipo2000 at a final concentration of 100 nM. Both flow cytometry and confocal microscopy were used to investigate the uptake efficiency of the liposomes in A549 cells. The results indicated that both CL and Lipo2000 effectively delivered siRNA to cells (Figure 2a), and significantly improved the uptake efficiency compared with free siRNA solution (Figure 2b).

3.3. Silencing of CYP1A1 gene expression in A569 lung cancer cell line

Transfection of induced A549 cells with CL-CYP1A1-siRNA caused a 7-fold down-regulation of CYP1A1 gene expression. Similar results were obtained with transfection using CYP1A1-siRNA Lipo2000 control. On the other hand, free (i.e. naked) CYP1A1-siRNA and negative control siRNA (NC siRNA) did not show any marked silencing effect

on CYP1A1 gene expression (Figure 3a). All agents were tested in the induced cells, and the unstimulated A549 cells were used as a negative control. The silencing effects of CL-CYP1A1-siRNA on CYP1A1 protein levels and enzymatic activity was also seen (Figure 3b and 3c), confirming successful retardation of gene expression target. CL-siRNA prepared using the film-coating proliposome technology caused a similar knockdown efficiency compared to the positive control Lipo2000. This clearly demonstrates that the facile approach of generating CL-siRNA using the proliposome technology was successful at providing a more stable powdered formulation than conventional liposomes. It was also capable of retarding the gene expression in levels similar to those of the established Lipo2000 transfection reagent.

3.4. Knockdown of CYP1A1 gene induces apoptotic cell death in 3MC- treated A549 cells

The number of apoptotic cells was quantified by FITC-Annexin V and propidium iodide (PI) double-staining. CL-siRNA triggered apoptosis in induced A549 cells (Fig.4a). 3-MC induced cells without further treatment were used as the negative control in these experiments in order to eliminate the inducer influence on the results. Findings revealed that 3-MC induction had a little impact on the cellular growth, whereas the induced cells

tended to undergo apoptosis with CYP1A1 silencing through the intrinsic apoptotic pathway marked by elevated caspase 3 and caspase 9, but not caspase 8, activities (Figure 4B), also confirmed by direct immunostaining (data not shown).

3.5. The Effect of CYP1A1 gene silencing on sphere formation in A549 lung cancer cell line

Sphere formation assay was performed to investigate the effect of CYP1A1 silencing on formation of spheroid colonies *in vitro*. Untreated induced A549 cells successfully produced spheroid colonies when cultured in a modified tumor sphere medium. On the other hand, spheres treated with CYP1A1 siRNA delivered by cationic liposomes or Lipo2000 formulation showed a suppressive effect on the formation of sphere colonies. The other groups including those untreated and mock did not exhibit this effect (Figure 5).

3.6. Antitumor efficacy of gene silencing of CYP1A1 in tumor-bearing nude mice using particulate-based proliposome technology

In order to investigate the impact of CYP1A1 silencing on tumor progression *in vivo*, we determined the antitumor efficacy of CL-siRNA in A549 xenograft nude mice model (Figure 6a). Results showed that growth rate of tumor with cationic liposome or Lipo2000

was significantly slower than that observed in the control groups including animals injected with PBS or naked (free) siRNA (Figure 6b/c). Moreover, the treatment caused down-regulation of the expression of CYP1A1 gene in the tumors as detected by RT-PCR on the third day after giving the intratumoral dose (Figure 6d). Thus, the reduction of CYP1A1 gene in induced A549 cells mediated by siRNA gave a significant tumor growth inhibition.

4. DISCUSSION

In this study we report that liposome-based gene delivery technology is a viable and stable approach for targeting the cancer causing gene CYP1A1. A major issue for liposomes is their instability as liquid dispersion, commonly when prepared using the thin-film hydration technique (Grit and Crommelin, 1993). This was overcome in the present study by using the film-coating proliposome technology to prepare powdered lipid formulations that, when needed, can be used to generate CL-siRNA complexes.

This technology, as demonstrated in our study, can potentially be considered for therapeutic intervention and treatment of lung cancer, one of the most common types of cancer and a leading cause of death (Torre et al., 2015). This approach comes as part of ongoing efforts to ameliorate the outcomes related to the undesirable pharmaceutical, pharmacokinetic and pharmacodynamic properties of lung cancer drugs, such as solubility, toxicity, stability, and

lack of selective effect on the cancerous cells (Tiwari et al., 2012). These properties can be enhanced by using drug vectors that are highly biocompatible and biodegradable (Zarogouldis et al., 2012).

Continuous exposure to tobacco smoking can induce the expression of CYP1A1, a gene present in extra hepatic tissues (Androutsopoulos et al., 2009), that is involved in the metabolic activation of PAH produced from tobacco smoking. After the induction, high CYP1A1 gene expression can contribute to the carcinogenic derivatives production and may initiate neoplastic transformation (Whitlock, 1999). Stimulated bronchial epithelial cells express high levels of CYP1A1 gene when induced by tobacco or environmental pollutants, predisposing them to lung cancer (Mercer et al., 2006). Hence, A549 human alveolar basal epithelial cell line represents a valuable model for the mechanistic studies involving induction of the pulmonary CYP system (Giard et al., 1973). In this study, we constructed a cell model on the basis of AhR mechanism through which CYP1A1 can be activated to a high level using 3-MC as previously reported (Hukkanen et al., 2000). In the induced cells, a high CYP1A1 gene expression was observed, similar to that seen in cancerous cells exposed to air contaminants. In our study, the 3-MC concentration was optimized to exhibit low toxicity and relatively high induction efficiency.

Limited studies have reported the relationship between inhibition of CYP1A1 gene and lung cancer therapy (Androutsopoulos et al., 2009). Flavonoid (such as quercetin), for example, was previously reported to inhibit CYP1A1 induction (Ciolino and Yeh, 1999). In the present work, we used RNAi as the inhibition strategy in lung cancer cells. Successful therapy using siRNA depends on effective delivery and protection against RNase. Owing to its large molecular weight and anionic nature, the uptake of siRNA by cancer cells is very poor, making the use of appropriate delivery systems highly advantageous (Gala et al., 2015). To overcome these issues, we prepared cationic liposomes via the lipid-coating proliposome technology shown previously to be suitable for large scale production (Gala et al., 2015). Using fluidized bed coating, the solid proliposomes produced can be stored at 18°C until needed for subsequent generation of liposomes, providing stability for several months (data not shown). In addition mass production and storage stability of proliposomes (as liposome precursors), the cationic liposomes were able to protect siRNA from nucleases and facilitated efficient transportation of siRNA into the cytoplasm, resulting in gene silencing effects similar to those exhibited by the commercially established Lipo2000. Indeed, both our *in vitro* and *in vivo* results indicated that CYP1A1 gene silencing by siRNA can regulate the cancer in the induced cells. Our data showed that the down-regulation of *CYP1A1* gene induced cellular apoptosis and interfered with the formation of tumor spheres *in vitro* and inhibited tumor development in BALB/c nude xenograft model.

Various murine models were established for the evaluation of novel therapeutics and examination of the molecular mechanisms underlying transformation, invasion and metastasis (Kellar et al., 2015). The A549 xenograft model was chosen in this study for the convenience of tumor measurement by making the cancer cells readily accessible (Kellar et al., 2015). Therefore *in vivo* results remain preliminary in nature and inconclusive. However, the emerging data confirm the validity of CL-siRNA-CYP1A1 as a proof of concept for targeting lung cancer, future experiments will explore different experimental designs including optimizing dosage and scheduling regimen to improve efficacy.

5. CONCLUSION

This study has shown that CYP1A1 gene can be a potential target for treatment of lung cancer. Cationic liposomes generated from film-coated proliposomes provided excellent siRNA carriers, with subsequent ability to silence the CYP1A1 gene both *in vitro* and *in vivo*. Further investigations to evaluate the aerosolization properties of CL-siRNA in animal models using the proliposome approach are warranted. This study will open the doors to further investigations in multiple therapeutic directions in the field of drug delivery and cancer treatment.

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DECLARATION OF CONFLICTS OF INTERESTS

The authors declare no conflicts of interests.

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FIGURE LEGENDS

FIGURE 1. Characterization of liposome-siRNA complex. (a) Scanning electron microscopy images of blank sorbitol and (b) Image of proliposome particles after coating with the lipid. (c) Transmission electron microscopy image of cationic liposomes generated from proliposomes. (d) Size and zeta potential of CL-siRNA complex at different cationic liposome to siRNA ratios. (e) The mobility of siRNA complexed with cationic liposomes at various molar ratios, ranging from 1-10 liposome to siRNA, by agarose gel retardation assay visualized by Goldview staining. (f) Stability of CL-siRNA complex against RNase. Cationic liposomes were complexed with siRNA at different molar ratios to study the degradation of siRNA by RNase by incubation with RNase at 37°C for up to 6 hours. The CL and siRNA N/P ratio was kept 4:1 in all samples and siRNA alone was used as negative control.

710

711 **FIGURE 2. Cellular uptake of CL-siRNA by A549 lung cancer cell line.** (a)
 712 Representative images of A549 cells transfected with FAM-siRNA, CL-FAM-siRNA or
 713 Lipo2000-FAM-siRNA. Cells were treated with 5 μ M 3-MC to induce CYP1A1 expression
 714 then incubated either with 100 nM liposome-free FAM-siRNA or 100 nM FAM-siRNA-
 715 liposome complexes in serum-free medium. After transfection, cells were stained with 4'6-
 716 diamidino-2-phenylindole (DAPI) and fluorescence images were taken by confocal
 717 microscope. (b) The cellular uptake efficiency of CL-siRNA in the induced cells was also
 718 measured by flow cytometry (n=3).

719

720 **FIGURE 3. Targeting of CYP1A1 gene using gene silencing approach.** A549 lung
 721 cancer cells were treated with 3-MC then transfected with CYP1A1-siRNA using liposome
 722 (CL/siRNA) or Lipofectomine 2000 (Lipo2000/siRNA). Non-stimulated A549 cells were
 723 used as a negative control whereas 3-MC stimulated A549 transfected with naked siRNA
 724 were used as a positive control. (a) Expression of CYP1A1 gene of was analyzed by
 725 quantitative RT-PCR using β -actin as internal control. Data are shown as normalized fold
 726 expression relative to the untreated control (n = 3), * p<0.05. (b) CYP1A1 enzyme activity
 727 was measured by Human CYP1A1 enzyme activity fluorescence quantitative detection kits

(n=3), * $p < 0.05$. (c) CYP1A1 enzyme content was detected with Human CYP1A1 ELISA kits (n=3), * $p < 0.05$.

FIGURE 4. Knockdown of CYP1A1 gene causes apoptosis in lung cancer cells. (a)

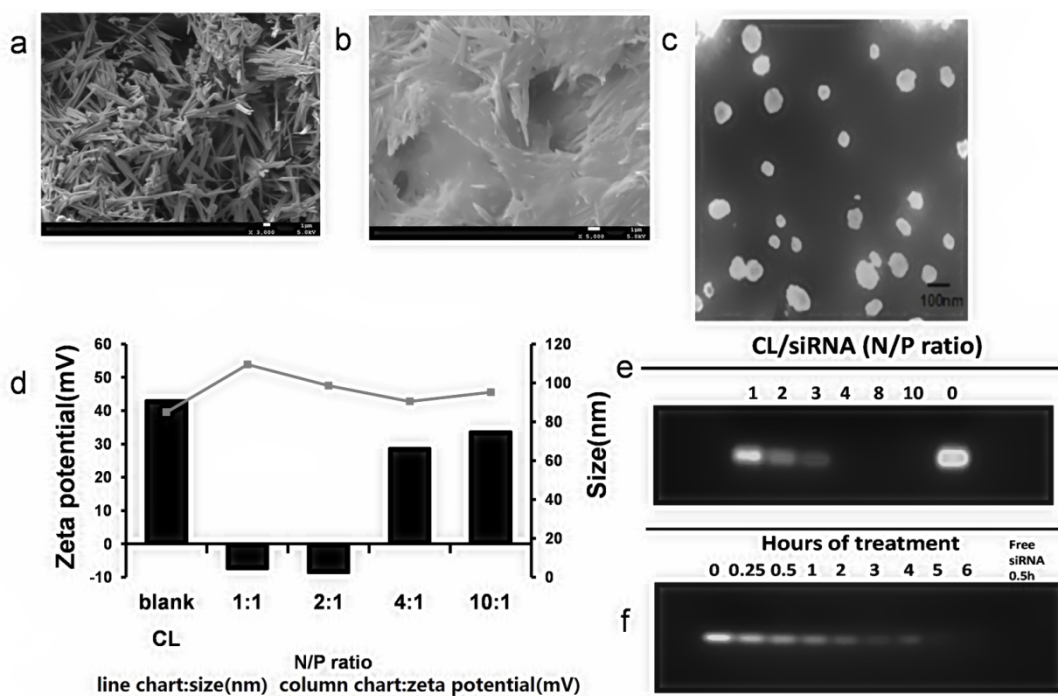
A549 lung cancer cells were treated with 3-MC for 24 hours then transfected with CL-CYP1A1-siRNA or CYP1A1 lipofectamine-2000. Cells were then stained with fluorescein-conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry. Percentages of apoptotic cells are presented as mean \pm SD (n = 3). (b) Quantification of the active caspase 3, caspase 8 and caspase 9 in 3-MC-induced A549 lung cells transfected with CL-CYP1A1-siRNA as performed by Flow Cytometry using caspase activity assay kits as described in the methods section. Data are presented as mean \pm SD (n = 3).

FIGURE 5. Effect of CYP1A1 gene silencing on A549-mediated spheroid colonies.

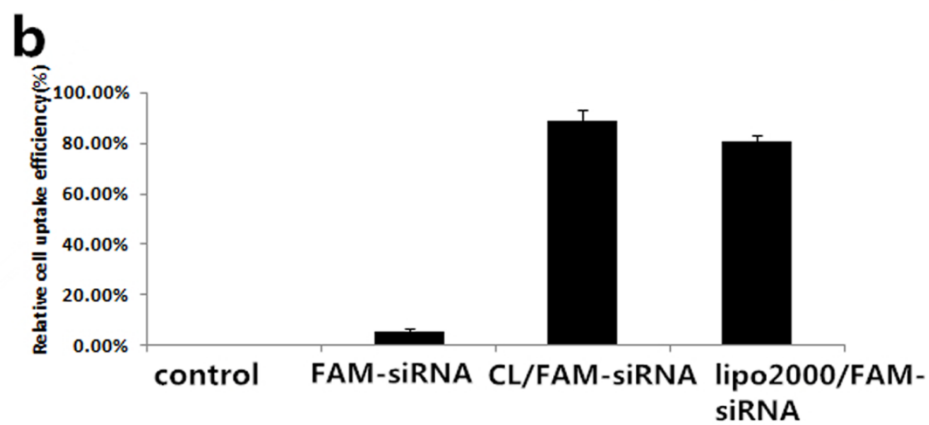
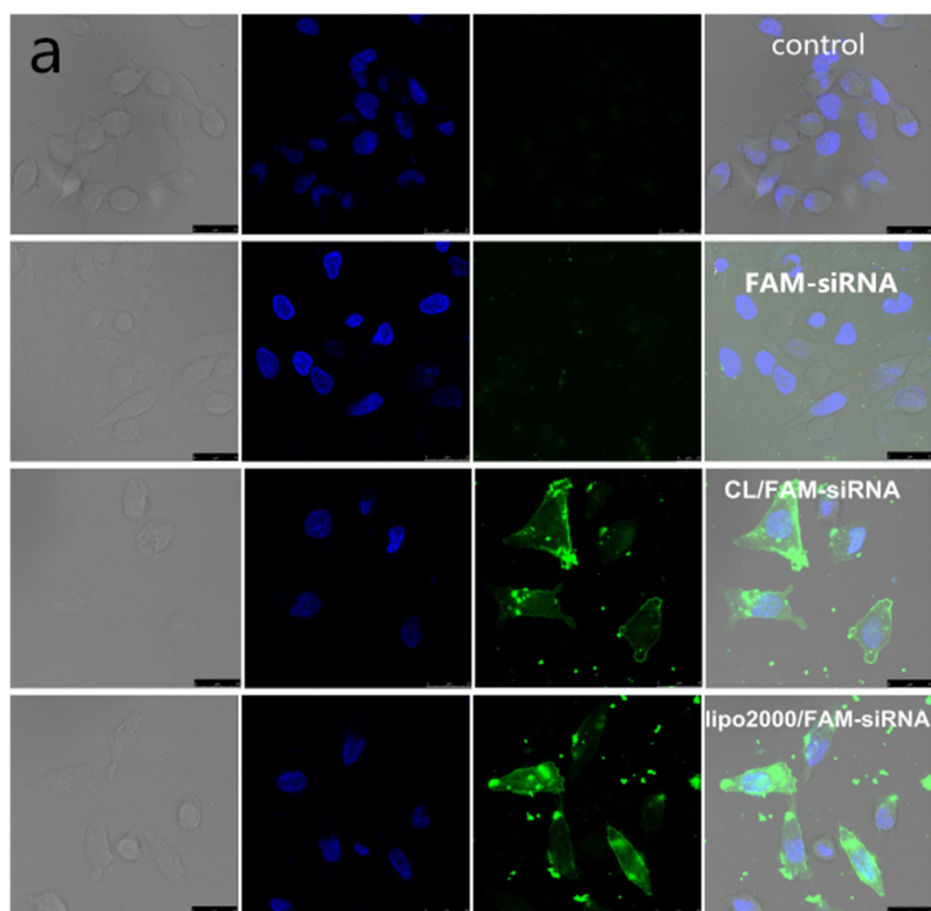
Spheroid colonies were generated as described in the methods section. Sphere cells were treated with 3-MC for 24 h and subsequently then transfected with CL-CYP1A1-siRNA or CYP1A1 lipofectamine-2000 for 72 h. Representative images shown are from three different experiments.

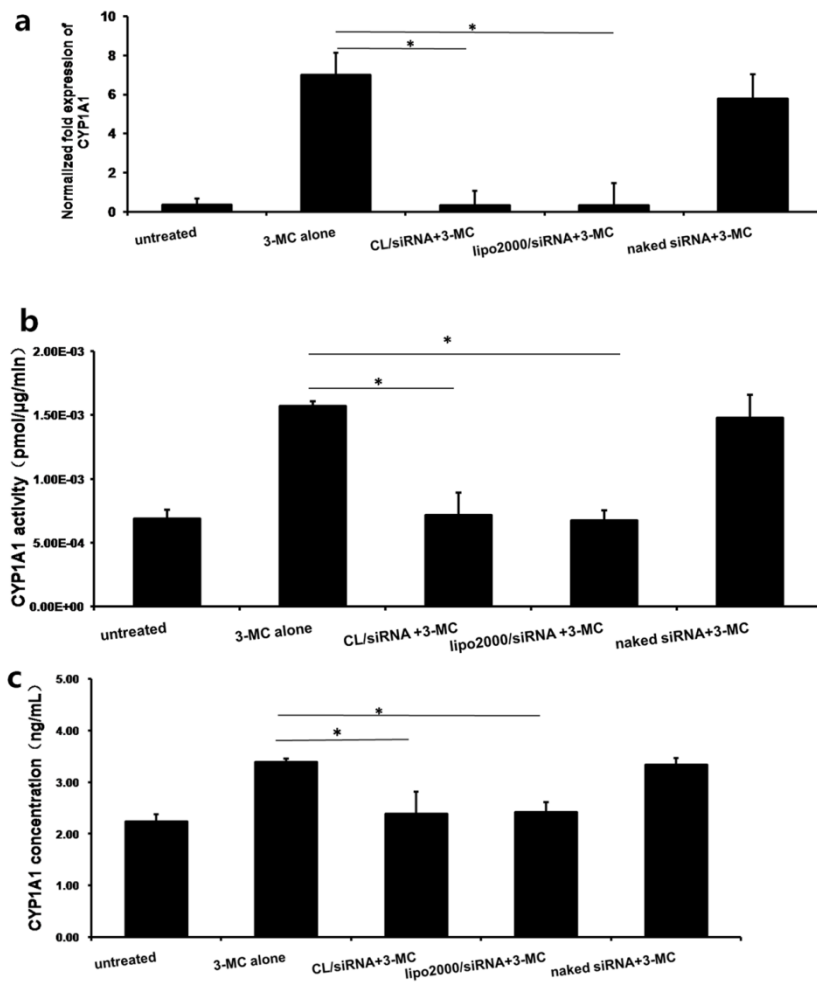
FIGURE 6. Inhibition of tumor growth using CYP1A1-siRNA in mouse model system.

BALB/c-nude mice were injected with 10 million A549 cells in serum free medium subcutaneously into right flank. The tumor bearing mice were divided into four treatment group (n=5). (a) All mice were injected with 3-MC and after 10 days were treated with (i) PBS (ii) CL-CYP1A1-siRNA (iii) Naked-siRNA and (iv) Lipofectamin-complexed CYP1A1-siRNA. (b) The volume of each tumor was measured at the indicated time points as described in methods. Results are expressed as mean, (n = 5), \pm SD. (c) Mice were sacrificed after 18 days with six intratumoural injection of CYP1A1 siRNA and images of each tumors were taken as shown (n=5). (d) Total RNA were isolated from tumor of each mice. Expression of CYP1A1 gene were quantified by RT-PCR (Data expressed as mean \pm SD; n=3).

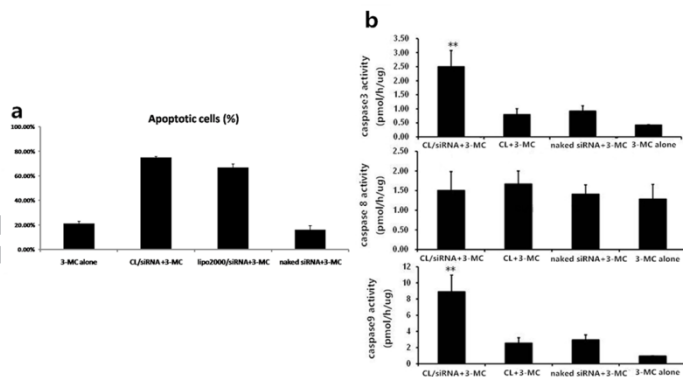


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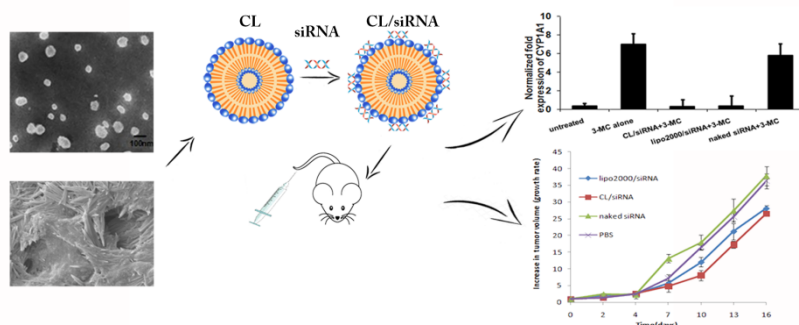
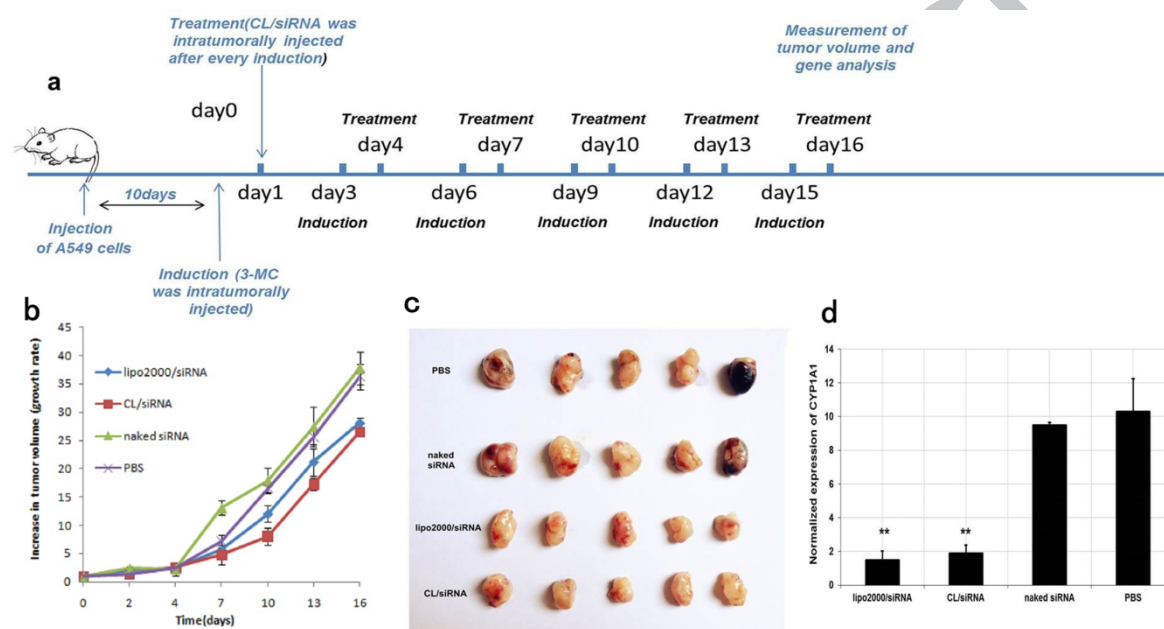


untreated

mock

CL/siRNA

Lipo2000/siRNA



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

768

769 ☐The authors declare the following financial interests/personal relationships which may be
770 considered as potential competing interests:
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